Cyclodextrins and Other Inclusion Complexation Approaches

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Introduction

Cyclodextrins are cyclic nonreducing oligosaccharides containing from six to twelve glycose units in a C-1 chair conformation, bonded through α-(1,4) linkages (**Figure 1**). The glycopyranose units are arranged in the shape of a hollow truncated cone. The larger opening of the molecule is surrounded by the secondary (C-2 and C-3) hydroxyl groups, while the primary (C-6) hydroxyl groups constitute the smaller end of the cone.

Since all the primary and secondary hydroxyl groups are located at the outside of the molecule, the exterior faces are hydrophilic. The interior cavity, essentially comprised of methylene linkages and glycosidic oxygen bridges, is relatively hydrophobic in comparison with polar solvents such as water. Furthermore, the glycosidic oxygen bridges produce a high electron density, giving the interior of the cavity a slightly Lewis-base character.

The three smallest cyclodextrin homologues are readily commercial available:

- α-cyclodextrin (cyclohexamylose);
- β-cyclodextrin (cycloheptamylose);
- γ-cyclodextrin (cyclooctamylose);

The basic property of cyclodextrins is their ability to form selective inclusion complexes with a broad variety of organic and inorganic molecules.

The formation of inclusion complexes is in general determined by the ability of the guest molecule to closely fit the cavity of the cyclodextrin. However, the polarity of the guest molecule also plays an important role.

Inclusion complexes are usually formed in the presence of water or in water mixed with organic modifiers.

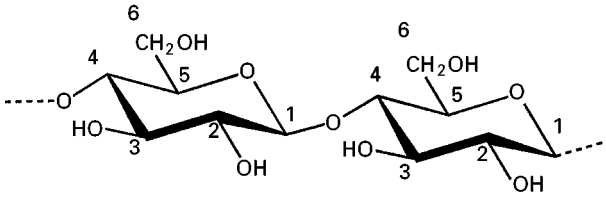

Figure 1 Numbering of carbon atoms in the cyclodextrin ring structure.

One of the first effective uses of cyclodextrins in chromatography was as mobile phase additive in thin-layer chromatography. In the mid-1980's a process was developed to produce stable cyclodextrin high performance liquid chromatographic (HPLC) phases. Nowadays, native α-, β- and γ-cyclodextrin, as well as a variety of derivatized cyclodextrin HPLC columns are commercially available. Also many cyclodextrin-based capillary gas chromatography (GC) columns are on the market. With the growing importance of capillary electrophoresis in chiral separations, the use of native cyclodextrin and cyclodextrin derivatives as an electrolyte additive steadily increases.

Cyclodextrins in HPLC Applications

Native cyclodextrin HPLC columns were deliberately designed to be used in the reserved-phase mode of operation, in order to take full advantage of the host–guest complexation capabilities of the molecule.

In a more recent and somewhat different experimental approach, the inclusion properties are suppressed by using a non-hydrogen bonding, polar organic solvent (e.g., acetonitrile) as the main component of the mobile phase. Acetonitrile has the tendency to occupy the cavity and seems to enhance hydrogen bonding between the hydroxyl groups on the cyclodextrin and hydrogen bonding groups on the chiral analyte.

In this so-called polar organic mode of operation, the addition of small amounts of glacial acetic acid and triethylamine is used as a tool to enhance enantioselectivity. On the other hand, the addition of a hydrogen bonding solvent such as methanol allows reduced retention of strongly retained molecules. This technique produces some unusual enantioselectivities that certainly enhance the usefulness of native cyclodextrin phases.

Furthermore, a variety of cylcodextrin derivatives has been immobilized on a chromatographic support, which can be used under normal as well as under reversed-phase conditions. The most popular commercially available derivatized cyclodextrin chiral selectors are listed in **Table 1**.

Mobile Phase Design and Parameter Optimization

Because complex stability constants usually have greater values in water or water–organic organic solvent mixtures than in a pure organic medium, native cyclodextrin columns are predominantly used in the reserved-phase mode. Ethanol, propanol, 1,4-dioxane, dimethyl sulfoxide, dimethyl formamide,

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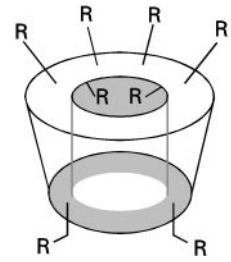
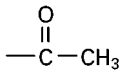
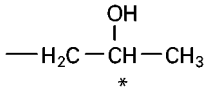
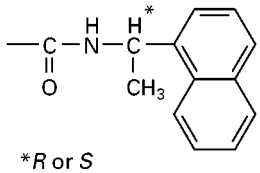
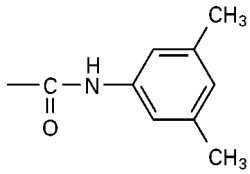
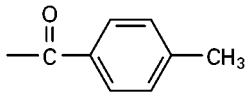
Table 1 Commercially available derivatized cyclodextrin phases



R	*Trade name, Advanced Separation Technologies*	
	Cyclobond® I 2000 Ac	Acylated
 **RS* or *S*	Cyclobond® I 2000 RSP Cyclobond® I 2000 SP	Hydroxypropyl ether
 **R* or *S*	Cyclobond® I 2000 RN Cyclobond® I 2000 SN	Naphtylethyl carbamate
	Cyclobond® I 2000 DMP	3,5-Dimethylphenyl carbamate
	Cyclobond® I 2000 PT	*p*-Toluoyl

methanol and acetonitrile have been used as organic modifiers. However, methanol and acetonitrile are most commonly used. It is difficult to predict in advance which of these two modifiers will produce the best separation in any given case. In many cases, pH and ionic strength of the aqueous part of the mobile phase are even more important than the choice of the organic modifier.

The following factors influence enantioselectivity.

Ionic strength With an eluent composed of a mixture of 5 millimolar tetrabutylammonium hydrogensulfate (TBAHS) in water and methanol in an 80–20 volume ratio, experiments were performed on a β-cyclodextrin Astec column (Cyclobond®) and a similar Merck column Merck (Chiradex®). The only difference between both stationary phases is the chemistry used to attach the β-cyclodextrin to the silica matrix.

In these initial experiments, especially on the Cyclobond® column, a poor peak shape for different products was observed. This effect was less pronounced on the Chiradex® column but it also occurred for some products. The origin of the poor peak shapes can have different causes, as for example an insufficient shielding of residual silanol groups. Therefore, the effect of the tailing reducer concentration was investigated.

Figure 2 illustrates the effect of the tailing reducer concentration on the retention factor and the resolution of miconazole (R14889). In this figure, the resolution factor based on the location of the valley point between two peaks is used. The resolution parameter (Auflösung (*9*)) is defined to evaluate the degree of separation between two partially resolved enantiomers. This measuring principle is based on the location of the valley point between two adjacent peaks. It is a useful and, from a measurement viewpoint,

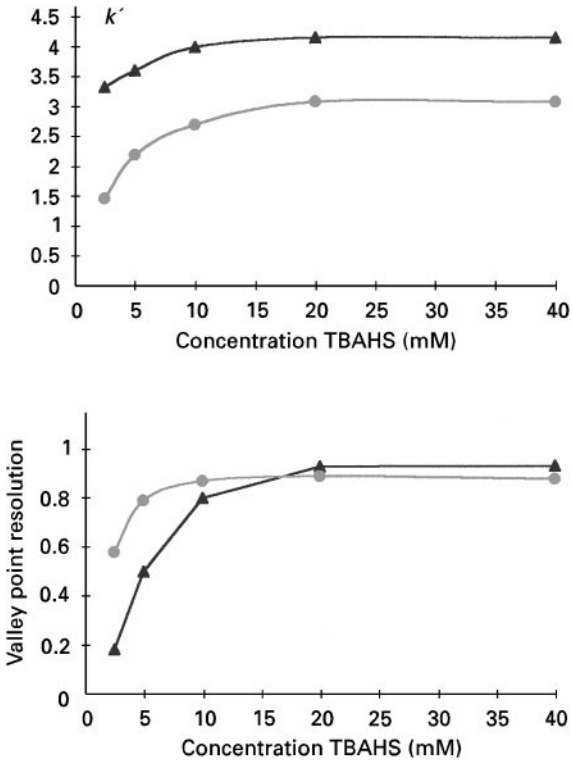
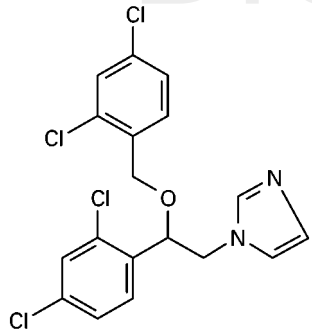


Figure 2 Influence or tailing reducer concentration on the retention factor and valley point resolution ▲, Cyclobond®; ●, Chiradex®. Experimental conditions: column: 25 cm × 4.5 mm ID chemically bonded cyclodextrin phase; mobile phase: TBAHS in water at different concentrations–methanol (80–20, v/v); flow rate: 1 mL min^{-1}. Solute:



easy evaluation technique for characterizing the separation degree between two peaks.

If we analyse **Figure 2**, it is easy to conclude that ionic strength has a strong effect on the chromatographic behaviour of the investigated chiral molecule. It is furthermore perfectly clear that the influence of ionic strength on the retention factor is more pronounced on the Chiradex® than the Cyclobond® column. However, for both types of stationary phase a constant value is observed above a tailing reducer concentration of 20 mmol. At low TBAHS concentration, the smallest resolution values are measured on the Cyclobond® column. Also, the ionic strength required to reach the highest resolution value is different for both columns.

Further experiments dealing with the effect of ionic strength on peak shape and chromatographic behaviour have demonstrated that for each individual column type–tailing reducer combination it can be very helpful to investigate this parameter thoroughly. However, to save time, we nowadays start our chiral method development work on cyclodextrin-based columns with tailing reducer concentrations between 30 and 50 mmol, because we have experienced that with these values there is in general a good chance of being successful.

Type of tailing reducer The acetic acid salt of triethylamine is popular as tailing reducer in reversed-phase chromatography. Instead of using acetic acid to adjust the pH value of an aqueous triethylamine solution, we investigated the usefulness of some other organic and inorganic acids. At first we did not expect any influence of the type of counterion on enantioselectivity, but some preliminary experiments showed a distinct effect, worthwhile to investigate further. Therefore, we examined about 30 products belonging to different product classes. As mobile phase, a mixture of 20 vol% of methanol and 80 vol% of 50 mmol aqueous triethylamine solution adjusted to a pH value of 2.5 with respectively hydrochloric, hydrobromic, phosphoric, perchloric, sulfuric, trifluoroacetic and oxalic acids was used.

For all the investigated products, the largest retention factors were observed for the triethylamine solution adjusted to the desire pH value with sulfuric and oxalic acids. Also with sulfate as counterion, the largest number of investigated products was partially or completely resolved. Compared to the other acids, with trifluoroacetic and perchloric acid a smaller number of products were separated. To further investigate the observed effect, we repeated the same experiments for a series of azoles, which differed only slightly in structure. For these substances, the effect of the counterion on resolution is illustrated in **Figure 3**. See also **Table 2**.

For this product series, the results confirm the initial observations. Furthermore, Figure 3 clearly demonstrates that even small change in molecular structure, as for example the number or position of the chorine atoms on one of the phenyl groups of the molecule, can have a tremendous effect on the enantioselectivity.

Although for the investigated series of azoles pH adjustment with sulfuric acid in general resulted in good peak shapes and high resolution values, one peculiarity was observed. Under these experimental

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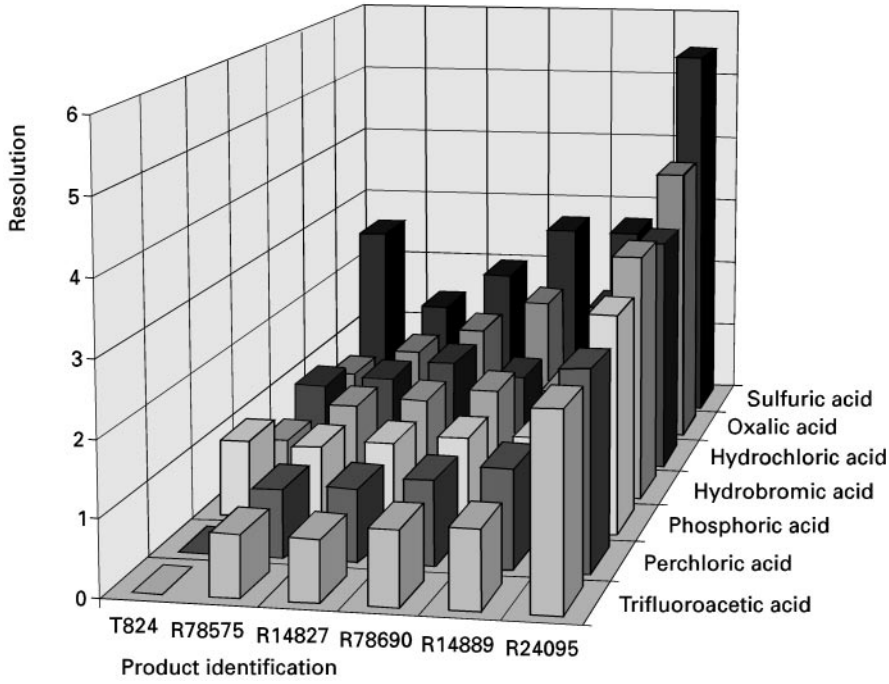


Figure 3 Azoles: effect of the counterion on resolution. Experimental conditions: column: 25 cm × 4.6 mm ID Cyclobond® (Astec); mobile phase: 50 mM triethylamine in water adjusted to pH 2.5 with different acids–methanol (80–20, v/v); flow rate: 1 mL min^{-1}, solutes; see Table 2.

conditions, R78575 was strongly retained and both enantiomers eluted as broad peaks with an irregular shape (**Figure 4**). On the other hand, pH adjustment of the triethlmaine solution with the other acids resulted in normal peak shapes (**Figure 5**). This is an indication that minor differences in structure, or small variations in experimental conditions, can have a tremendous effect on the chromatographic behaviour of enantiomers.

A comparable set of experiments was subsequently performed using tetrabutylammonium hydroxide instead of triethylamine as tailing reducer. In this set of experiments, oxalic acid was excluded and perchloric acid could not be used owing to the formation of an insoluble salt with tetrabutylammonium hydroxide in the water–methanol mixture. For the whole test series of 24 different substances, sulfuric acid could be identified as the counterion that generated the highest resolution values. From a practical point of view, it is certainly an advantage that sulfate is the anion of choice. For temperatures between room temperature and 50°C the corrosion of stainless steel (316 or equivalent) is negligible for dilute sulfuric acid solutions, while the chemical resistance of this material for chloride ions (even in low concentrations), is rather limited.

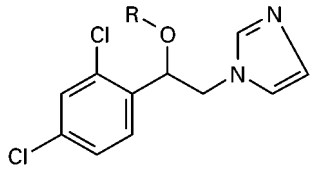
In conclusion, the anionic part of the tailing reducer has a clear effect on enantioselectivity, as illustrated previously. The influence of the counterion on the retention factor and resolution of the investigated compounds can possibly be related to a difference in ability of the counterion to compete with the organic guest molecule for the hydrogen bonding sites of the cyclodextrin host.

To complete the experiments concerning the influence of the type of tailing reducer, we also found it necessary to do some tests in which the anionic part of the tailing reducer was kept identical while the cationic part was varied. In these experiments, a 30 mmol solution of tetramethyl-, tetraethyl-, tetrapropyl- and tetrabutylammonium hydroxide solution was adjusted with sulfuric acid to a pH value of 2.5 and used as mobile phase in an 80–20 volume ratio with methanol as organic modifier.

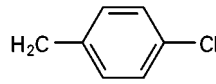
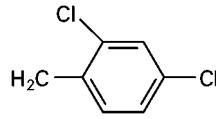
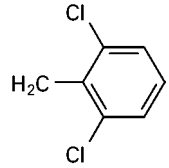
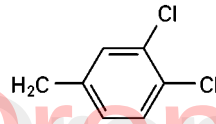
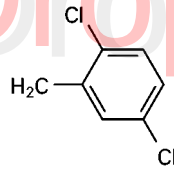
The smallest resolution values are observed for tetramethylammonium hydroxide and the highest values for tetrabutylammonium hydroxide, but compared with the influence of the anionic part of the tailing reducer the differences are far less pronounced. However, for the investigated test series, the tailing factor measured at 10% of the peak height gradually decreases with increasing chain length of the alkylammonium hydroxide.

pH Whatever type of silica-based reversed phases are used, these materials always display some acidic

Table 2 Structures of the investigated azole derivatives



Azoles

Product	*R*
T824	H
R14827 (econazole)	
R14889 (miconazole)	
R24095 (isoconozole)	
R78575	
R78690	

surface area properties owing to residual hydroxyl groups on the silica surface. During the analysis of basic substances not only hydrophobic interactions take place, but also acid–base interactions between these acidic groups and basic functions in the analyte can be expected to occur. This type of interaction often results in increased retention combined with peak tailing. One of the possibilities to solve this problem is to adjust the pH below the pK_a value of the sample that has to be analysed. At pH values lower than pK_a – 2 the basic function is protonated and a salt is formed which no longer has basic properties.

Because the cyclodextrin columns are used under reversed-phase conditions, it is certainly worthwhile to investigate the effect of pH on enantioselectivity. For some monobasic molecules, the effect of pH on the retention factor is depicted in **Figure 6**.

Figure 6 clearly illustrates that the retention factor of the investigated products follows a typical reversed-phase pattern. At higher pH values the degree of protonation of the analyte diminishes. As a result the chance for hydrophobic interactions with the stationary phase increases and higher retention values are measured. When the pH is equal to the pK_a value of the sample, the number of protonated and nonprotonated molecules is equal. Small changes in pH around this value will immediately have an effect on the retention factor. Even the effect of small structural changes, causing some differences, in hydrophobic nature of the nonprotonated solute molecules, can be clearly observed.

R8110 has the smallest retention factor owing to the presence of a fluorine atom on the phenyl group, giving the molecule a more polar character than the other two members of the test series. The largest retention factors are measured for R7405 bearing an ethyl group on the ester function attached to the imidazole ring instead of a methyl group for R7315.

To better visualize the effect of pH variations on enantioselectivity we often use for the graphical

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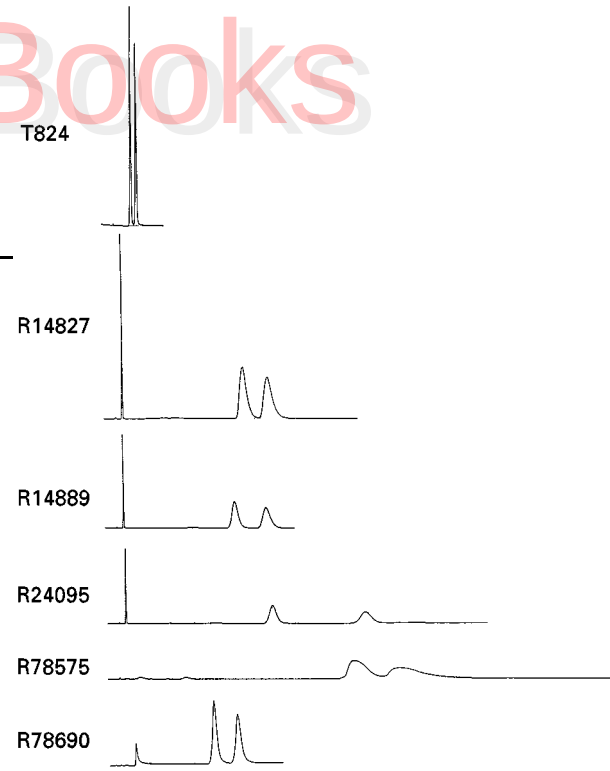


Figure 4 Peak shape of the different azoles. Experimental conditions: column: 25 cm × 4.6 mm ID Cyclobond® (Astec); mobile phase: 50 mM triethylamine in water adjusted to pH 2.5 with sulfuric acid–methanol (80–20, v/v); flow rate: 1 mL min^{-1}; solutes: see Table 2.

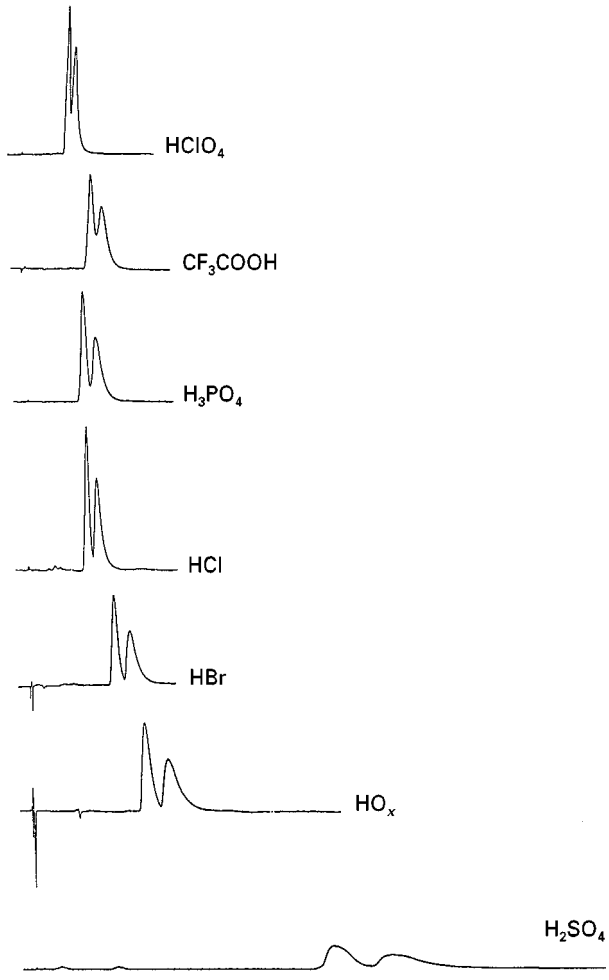
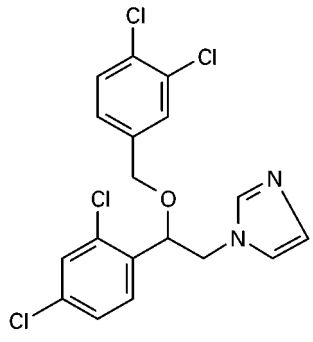


Figure 5 Peak shape of R78575. Experimental conditions: column: 25 cm × 4.6 mm ID Cyclobond® (Astec); mobile phase: 50 mM triethylamine in water adjusted to pH 2.5 with different acids–methanol (80–20, v/v); flow rate: 1 mL min^{-1}; solute:



representation of experimental data the degree of protonation of the solute molecules instead of pH values. For monobasic substances the ratio between protonated and nonprotonated molecules at a certain pH value can be easily calculated:

$$R\text{–}NH_3^+ \leftrightarrow R\text{–}NH_2 + H^+$$

$$K_a = \frac{[R\text{–}NH_2]\cdot[H^+]}{[R\text{–}NH_3^+]}$$

$$pK_a = pH + \log\frac{[R\text{–}NH_3^+]}{[R\text{–}NH_2]}$$

(Henderson–Hasselbalch equation)

$$pK_a - pH = \log\frac{[R\text{–}NH_3^+]}{[R\text{–}NH_2]}$$

From:

$$10^{[pK_a - pH]} = \frac{[R\text{–}NH_3^+]}{[R\text{–}NH_2]}$$

and:

$$[R\text{–}NH_3^+] + [R\text{–}NH_2] = 100$$

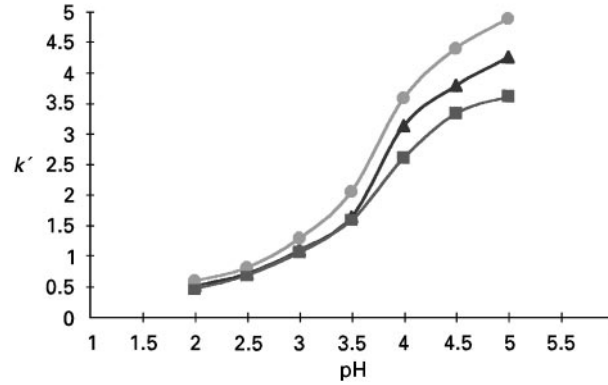
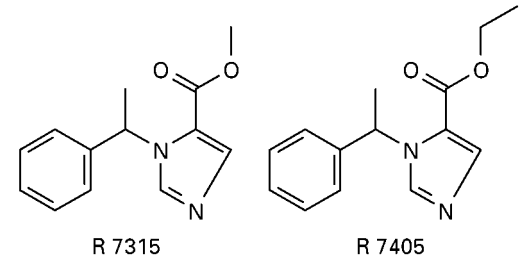
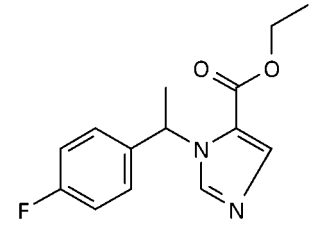


Figure 6 Effect of pH on rentention factor. ▲, R7315; ●, R7405; ■, R8110. Experimental conditions: column: 25 cm × 4 mm ID Chiradex® (Merck); mobile phase: 50 mM triethylamine in water adjusted to different pH values with sulfuric acid–methanol (80–20, v/v); flow rate: 1 mL min^{-1}; solutes:



R 7315 R 7405



R 8110

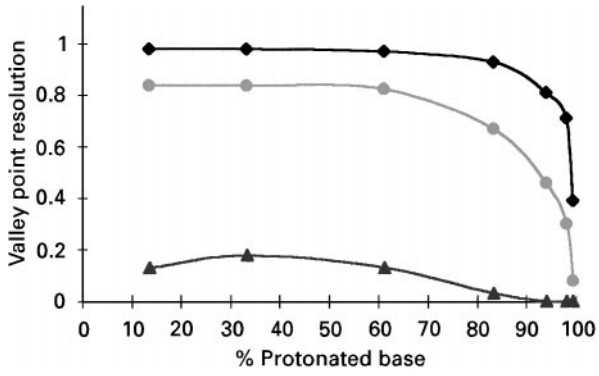
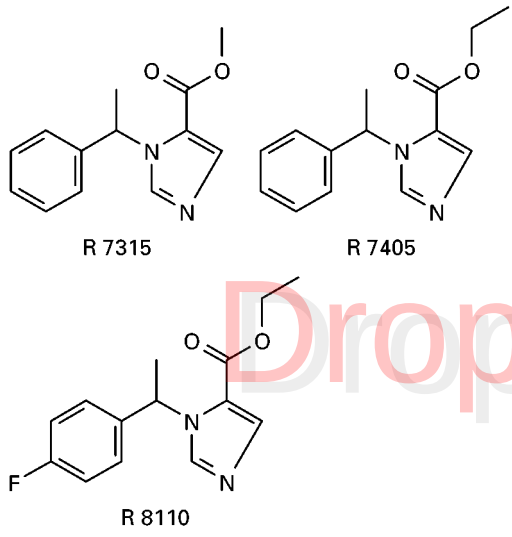


Figure 7 Effect of degree of protonation of the analyte on resolution. ◆, R7315; ●, R7405; ▲, R8110. Experimental conditions: column: 25 cm × 4 mm ID Chiradex® (Merck); mobile phase: 50 mM triethylamine in water adjusted to different pH values with sulfuric acid–methanol (80–20, v/v); flow rate: 1 mL min^{-1}; solutes:



it is possible to easily calculate for each pH value the ratio between protonated and free base. For R7315, R7405 and R8110 the valley point resolution versus the percentage protonation is depicted in **Figure 7**.

For the investigated product series, it is clear that under the experimental conditions applied above a certain degree of protonation of the solute molecules the enantioselectivity strongly decreases. Probably due to an increase in hydrophilic character of the protonated molecules, the strength of the hydrophobic interactions between the analyte and the cavity of the cyclodextrin molecule diminishes, with a reduced enantioselectivity as a result. Within the investigated pH range, triethylamine ($pK_a = 10.72$) always remains fully protonated. Therefore, competition for hydrophobic interactions with the cyclodextrin cavity between the tailing reducer and the solute molecules has to be considered as nonexistent and can be excluded as a possible reason for reduced enantioselectivity at higher pH values. Besides investigations on the effect of pH using triethylamine as basic substance in the aqueous part of the mobile phase, experiments have also been performed with different tetraalkylammonium hydroxides adjusted to the desired pH value with sulfuric acid. Within the same pH range, tetrabutylammonium hydroxide and triethylamine displayed comparable patterns when the valley point resolution was plotted against the percentage of protonation of the solute molecules.

For a dibasic substance (R60844) with respectively pK_a values of 5.4 and 6.7 the protonation pattern versus pH, together with the valley point resolution for two types of mobile phase additives, is given in **Figure 8**.

Although for the dibasic product R60844 both basic functions remain fully protonated up to a pH value of 4, the resolution continuously increases between pH 2 and 4. However, above pH 4 the enantioselectivity rapidly decreases.

For the different product series that have been examined we have observed that under the same experimental conditions, in general, higher resolution values are obtained with triethylamine than with

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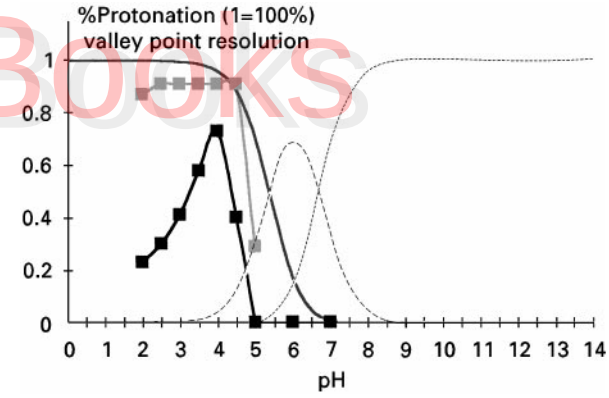
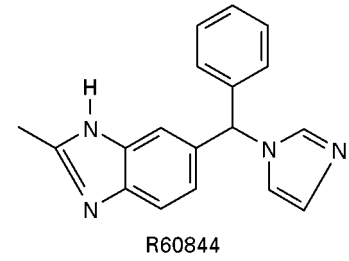


Figure 8 Effect of pH on the degree of protonation and resolution of a dibasic substance. —, Alpha 0; – – –, alpha 1; - - - -, alpha 2; ■, TBA-OH; ■, TEA. Experimental conditions: column: 25 cm × 4 mm ID Chiradex® (Merck); mobile phase: 50 mM triethylamine in water adjusted to different pH values with sulfuric acid–methanol (80–20, v/v); and 30 mM tetrabutylammonium hydroxide in water adjusted to different pH values with sulfuric acid–methanol (80–20, v/v); flow rate: 1 mL min^{-1}; solute;



tetrabutylammonium hydroxide as mobile phase additive.

In conclusion, the stability of the inclusion complexes formed between the solute molecule and the cyclodextrin seem to be dependent on the charge of the guest molecule. Therefore, the retention as well as the degree of separation of molecules bearing an ionizable acidic or basic group can be affected by changing the pH. However, the effect of pH variations is not so easy to predict.

In the experiments performed, some products (R7315, R7405, T824, etc.) display the highest resolution values when they are present as free base or as a partially charged molecule. Other products (R60844, most of the azoles) reach maximum resolution when they are completely or nearly completely protonated. For some products the effect of pH variations within a rage of 2–3 pH units is small, while for others extreme effects can be observed. Furthermore, for different types of tailing reducers the resolution versus pH profiles can have a different shape.

Therefore, the different experiments that have been performed to investigate the effect of pH on enantioselectivity clearly demonstrate that besides host–guest complexation interactions between the solute molecule and the cyclodextrin cavity, the hydroxyl groups at the outside of the cyclodextrin molecule together with reversed phase and other less predictable types of interactions certainly play an important role in the chiral recognition process.

Because small changes in pH can have a tremendous influence on the enantioselectivity, it is certainly advisable to thoroughly investigate this parameter during method development and optimization work on cyclodextrin columns.

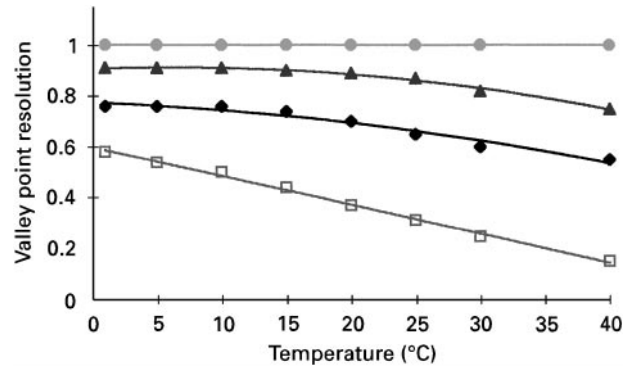
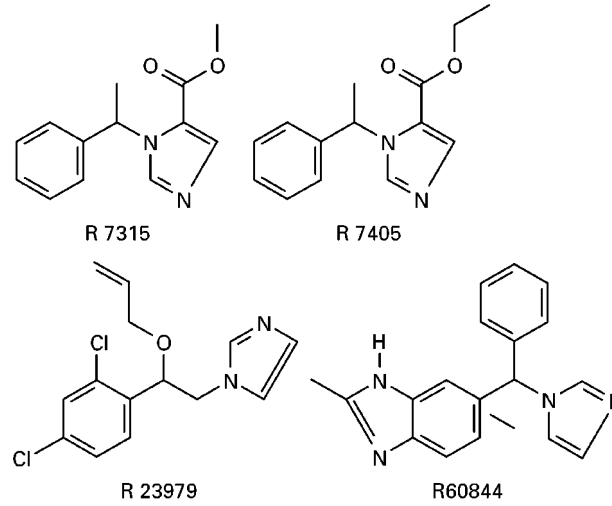


Figure 9 Influence of temperature on the retention factor. ◆, R7315; □, R7405; ▲, R60844; ●, R23979. Experimental conditions: column: 25 cm × 4 mm ID Chiradex® (Merck); mobile phase: 30 mM triethylamine hydroxide in water adjusted to different pH values with sulfuric acid–methanol (80–20, v/v); flow rate: 1 mL min^{-1}; temperature range: 1–40°C; solutes:



Temperature In equilibrium-based processes, temperature plays an important role. For all investigated compounds, a very good linear relationship between the natural logarithm of the retention factor and the inverse of temperature is observed. For the products R7315 and R7405 only small differences in slope are measured. Indications that for both products the enthalpy values for solute–stationary phase transfer are very similar. The effect of temperature on the valley point resolution is given in **Figure 9**.

Only for R23979 was a valley point resolution of one measured for the whole temperature range between 1 and 40°C meaning that for this product the effect of temperature variation is of the same magnitude for both enantiomers. For R60844 and R7315 practically no influence on the resolution was observed between 1 and 15°C. Above that temperature, for both products the resolution value starts to decrease in a similar way. The continuous decrease of the resolution value with increasing temperature observed for R7405 can be considered as a logical behaviour, because for R7405 and R7315 the smallest retention factors are measures – an indication that the binding forces between the stationary phase and these solutes are smaller than for the other products investigated. As a result, the effect of a temperature increase on the resolution is more pronounced for these compounds. However, we did not expect to observe a different effect of temperature variations for R7315 and R7405, because for both products only minor differences in enthalpy values were measured. Therefore, temperature variations probably have the same influence on both enantiomers of R7405, while the enantiomers of R7315 are affected in a different way.

In conclusion, temperature variations have an influence on the retention factor as well as on the resolution value. However, the effects observed differ from one product to another. Therefore, this parameter

has to be investigated on an individual basis during method development and optimization work.

Type and concentration of organic modifier It is known that for the reversed-phase analysis of several alkylbenzenes on chemically bonded cyclodextrin columns the type of alcohol, together with its concentration in the mobile phase, strongly influences the retention behaviour of these substances.

To investigate the effect of type of alcohol on enantioselectivity, we performed some experiments in which the normally used modifier (methanol) was replaced by the same amount of ethanol 1-propanol. For all the investigated substances, the retention factor strongly decreased with increasing chain length of the alcohol used.

The retention factors measured for an ethanol-based mobile phase are about 30–50% lower than the values observed for methanol as organic modifier. A decrease of approximately the same magnitude could be observed when ethanol was replaced by 1-propanol. Therefore, in extreme cases the retention factor drops to about 20% of the value measured for methanol, when this solvent is replaced by the same amount of 1-propanol. With increasing chain length the hydrophobicity of the alcohol increase, which enhances the chance for competition between the solvent and the analyte molecules to interact with the hydrophobic cyclodextrin cavity.

Because a strong decrease in retention time of the solutes could be observed when ethanol or 1-propanol was used as organic modifier, these solvents seem to have a greater affinity for the cyclodextrin cavity than methanol. Therefore, they will be more effective for displacing strongly retained substances from a cyclodextrin column. The manufacturers of cyclodextrin columns in fact use this property, because they recommend regenerating their columns by passing several column volumes of pure ethanol through the column, followed by pure water and then methanol.

In general, an increasing water content in the mobile phase increases both the retention and the enantioselectivity. However, for practical applications the retention factors are generally too long when mobile phases with a high water content are used, and a compromise has to be found. For our product classes we therefore use a mixture of 80 vol% of aqueous phase and 20 vol% of methanol as a typical starting mobile phase composition. If the products are too strongly retained or do not elute at all, the amount of methanol is systematically increased.

Flow rate Due to the very specific type of interactions, which play a major role in chiral recognition processes, chiral stationary phases often display slow mass transfer characteristics. Therefore, on chiral stationary phases, flow rate can have a strong effect on the enantioselectivity. For difficult separations, lowering the flow rate certainly has to be considered as a tool to enhance enantioselectivity.

Derivatized Cyclodextrins

Native cyclodextrin columns cannot be used effectively for the separation of enantiomers under normal phase chromatographic conditions. On the other hand, different naturally occurring chiral molecules that have been derivatized are extensively and very successfully used in the normal phase mode of operation.

Triacetylcellulose, obtained by heterogeneous acylation of cellulose, was one of the first commercially available derivatives. However, the later developed and commercialized aromatic cellulose and amylose derivatives (benzoates, carbamates), compared with triacetylcellulose, are much more universally applicable. Owing to the broad applicability of the cellulose and amylose derivatives similar cyclodextrin-based stationary phases have been developed.

In our laboratories we investigated the possibilities of the derivatized cyclodextrin columns under normal as well as reversed-phase chromatographic conditions. We also compared these phases with the corresponding cellulose derivatives.

Our first experiments on the functionalized cyclodextrin phases were performed under normal phase conditions. A series of 42 products covering a broad range of organic chemistry were investigated on the *S*-naphthylethyl carbamate, the *para*-toluoyl and 3,5-dimethylphenyl carbamate cyclodextrin derivative, using n-hexane-2-propanol in different ratios as the mobile phase.

The results obtained were rather poor. Of the whole test series only six products were partially or completely resolved on the 3,5-dimethylphenyl carbamate column. The situation was even worse on the two other columns tested. Therefore, we decided to switch immediately to the reversed-phase mode of operation. The initially used test series of 42 products was first investigated on the three above-mentioned cyclodextrin columns with a mobile phase consisting of 0.5% ammonium acetate in water and methanol in a 30–70 volume ratio. This mobile phase composition was chosen after some preliminary experiments, which demonstrated that higher water content caused a tremendous increase in retention times. Compared with the results under normal phase conditions, the number of products separated and the degree of separation were much better on all the investigated

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column types. Because earlier experiments on the native cylcodextrin phases have demonstrated that an acidic pH generally results in better separations, a 20 mM tetrabutylammonium hydrogensulfate solution (pH 2.3) was thereafter used as tailing reducer.

Owing to the well-known reversed-phase effect of retention decrease with lowering pH values, the methanol content in the mobile phase had to be reduced to 40 vol% instead of the 80 vol% used in the experiments with ammonium acetate as tailing reducer. Under these experimental conditions, the largest number of products was separated on the *S*-naphthylethyl carbamate column, although the results on the other two columns only differed slightly. It was also interesting to observe that some products, which could not be separated on native cyclodextrin, were completely resolved on one of the derivatized phases. In the next set of experiments, a 20 mM solution of respectively tetramethyl-, tetraethyl- and tetrabutylammonium hydroxide was adjusted with sulfuric acid to a pH value of 2.5 and used in combination with 70 vol% of methanol as the mobile phase. The effect of the cationic part of the tailing reducer is not clear. With tetraethylammonium hydroxide the largest number of products are partially or completely resolved, but the resolution values are in general somewhat higher with tetramethylammonium hydroxide, although the differences with the two other alkylammonium hydroxides are minimal. Only for one member of the test series was tetramethylammonium hydroxide required to obtain partial resolution.

On native cyclodextrin columns, we could clearly demonstrate the influence of the anionic part of the tailing reducer. Therefore, a similar test was done on the 3,5-dimethylphenyl carbamate cyclodextrin derivative, using 20 mM tetramethylammonium hydroxide adjusted to pH 2.5 with respectively trifluoroacetic, hydrochloric, phosphoric, (*d*)-camphorsulfonic and sulfuric acids, (*d*)-Camphorsulfonic acid has been deliberately chosen to investigate eventual additional effects by introducing chirality in the mobile phase. After pH adjustment, the aqueous phase was mixed with methanol in a 30–70 volume ratio. Some products were also tested with a 50–50 mixture of aqueous phase and methanol. Comparable with the observations on the native cylcodextrin column, and also on the functionalized cyclodextrin, pH adjustment with sulfuric acid resulted in the largest number of separations. For all the other acids, the number of partially or completely resolved products dropped to about 50% or less of the value observed for sulfuric acid. However, three products which could not be separated with one of the different acids tested were partially resolved when (*d*)-camphorsulfonic acid was used to adjust the pH of the tetramethylammonium hydroxide solution.

Comparison of derivatized cyclodextrins and the corresponding cellulose derivatives Because derivatized cellulose and amylose columns are extensively used in our laboratories for both analytical and preparative chromatographic applications, it seemed worthwhile to compare these phases with the corresponding cyclodextrin derivatives. At present only two cellulose phases are commercially available which can be used equally well under reversed-phase and normal phase conditions, namely Chiralcel® OD-R (3,5-dimethlyphenyl carbamate and Chiralcel® OJ-R (*para*-methylbenzoate) (Daicel, Japan). We compared these phases with Cyclobond®-DMP and Cyclobond®-PT columns (Advanced Separation Technologies), respectively.

The first experiments were performed under normal phase conditions, using n-hexane–2-propanol in a 70–30 ratio as the mobile phase. If products eluted too fast with this mobile phase composition, the amount of 2-propanol was reduced to respectively 20 or 10 vol%. A total of 21 different products were examined. The results of these experiments are summarized in **Table 3**.

From this data it is clear that for the investigated product classes the cellulose derivatives are far superior compared to the corresponding cyclodextrin phases when normal phase conditions are applied. We thereafter examined the same product series under reversed-phase conditions using a mixture of 70 vol% of methanol and 30% of a 50 mM triethylamine solution adjusted to pH 2.5 with sulfuric acid. The results of these experiments are summarized in **Table 4**.

When we compare this data with the results obtained under normal phase conditions, we have to conclude that in the reversed phase mode of

Table 3 Derivatized cyclodextrins versus the corresponding cellulose derivatives under normal phase conditons

Column type	*Good separation* $\vartheta > 0.90$		*Partially resolved*[a]		*Total*	
	Number	*%*	*Number*	*%*	*Number*	*%*
Chiralcel OD-R	9	42.9	6	28.6	15	71.4
Cyclobond I-DMP	2	9.5	3	14.3	5	23.8
Chiralcel OJ-R	13	61.9	5	23.8	18	85.7
Cyclobond I-PT	–	–	2	9.5	2	9.5

[a]For the partially resolved peaks, the resolution on the cellulose derivatives is always much higher than on the cyclodextrin derivatives.

Table 4 Derivatized cyclodextrins versus the corresponding cellulose derivatives under reversed phase conditons

Column type	*Good separation $\vartheta > 0.90$*		*Partially resolved*[a]		*Total*	
	Number	*%*	*Number*	*%*	*Number*	*%*
Chiralcel OD-R	2	9.5	10	47.6	12	57.1
Cyclobond I-DMP	2	9.5	5	23.8	7	33.3
Chiralcel OJ-R	14[b]	66.7	–	–	14	66.7
Cyclobond I-PT	–	–	3	14.3	3	14.3

[a]For the partially resolved peaks, the resolution on the cellulose derivatives is always much higher than on the cyclodextrin derivatives.

[b]All products are fully baseline resolved ($\vartheta = 1.00$).

operation fewer products are separated on the cellulose derivatives, although on the Chiralcel® OJ-R column all separated products are fully baseline resolved. The smallest resolution value equals 2.5 while the largest value is greater than 12.5, while under normal phase conditions the highest resolution value observed equals 6.3.

On the cyclodextrin derivatives a few more products are separated but the increase in number is certainly not spectacular. Furthermore, in many cases where partial resolution has been indicated in the table the chromatograms only showed a small deviation in the peak shape, indicating the early beginning of separation.

For a series of products which under comparable experimental conditions are all very well separated on the native cyclodextrin column, the results on the different cyclodextrin and cellulose derivatives using 50 mM triethylamine adjusted to pH 2.5 with sulfuric acid and methanol in a 30–70 volume ratio as mobile phase are illustrated in **Figure 10**.

Because only one substance of the test series is separated on the Cyclobond-PT column, and most of the products are only partially resolved on the Cyclobond-DMP column, while all these products are perfectly baseline resolved on native cyclodextrine, it is clear that other parameters must play a role in the chiral recognition process on the derivatized phases.

As a general conclusion it can be stated that for the type of substances investigated the derivatized cyclodextrin columns are, in both modes of operation (normal as well as reversed phase), less universally applicable than the corresponding cellulose derivatives.

Hydroxypropyl-β-Cyclodextrin Derivative

A hydroxypropyl-β-cyclodextrin column (experimental phase of the Chromatography Research group of Merck Darmstadt) in the reversed-phase mode of operation has been extensively investigated. The result on this type of material were completely comparable with the data obtained on the native cyclodextrin columns. However, for the whole range of products the degree of separation was in general

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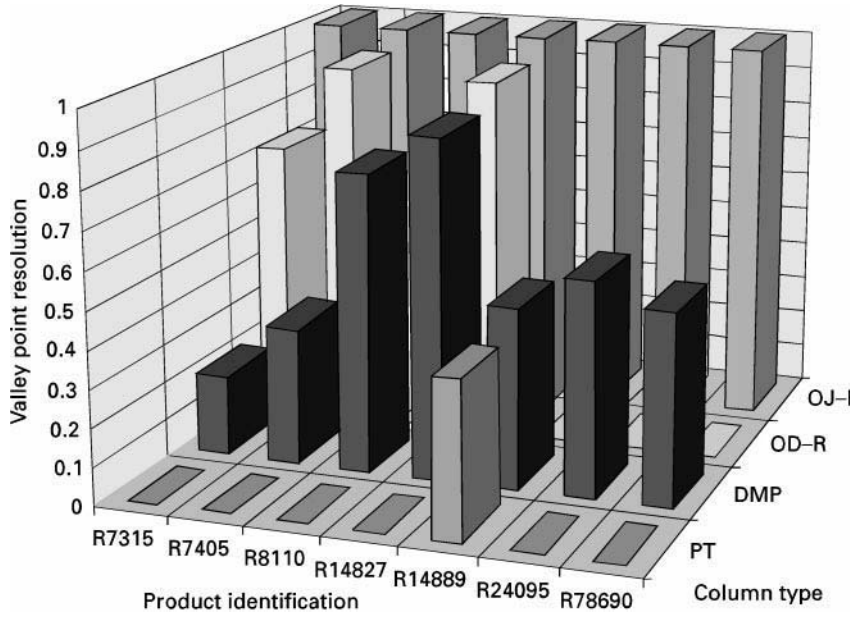


Figure 10 Derivatized cylcodextrin columns versus the corresponding cellulose derivatives. Experimental conditions: column: 250 mm × 4.6 mm ID (Cyclobond®-DMP, Cyclobond®-PT, Chiralcel® OD-R, Chiralcel® OJ-R); mobile phase: 50 mM triethylamine adjusted to pH 2.5 with sulfuric acid–methanol (30–70, v/v); flow rate: 1 mL min^{-1}.

better on the hydroxypropyl column. To investigate new products, we therefore always start our experiments on a hydroxypropyl-β-cyclodextrin column instead of using the classical native β-cyclodextrin type of material.

γ-Cyclodextrin

A test series of 28 products, which also have been investigated on β-cylcodextrin, have been examined on a γ-cyclodextrin column using a mobile phase consisting of 80 vol% of a 50 mM triethylamine solution in water adjusted to pH 2.5 with sulfuric acid and 20 vol% of methanol. On the β-cyclodextrin column, 22 products were partially or completely resolved. On the γ-derivative only nine products could be resolved.

Compared with the results obtained on the β-cyclodextrin column, it is clear that from a general point of view the γ derivative is less suitable for the separation of the investigated product series. Nevertheless, it remains an additional tool that might help to solve a separation problem when experiments on other types of cyclodextrin columns fail.

Dynamically Generated Cyclodextrin Phases

Instead of using the commercially available chemically bonded cyclodextrin phases, it is also possible to perform enantiomer separations on a reversed-phase column after addition of cyclodextrin or cyclodextrin derivates to the mobile phase. A number of experiments have been performed with hydroxypropyl-β-cyclodextrin as mobile phase additive. This derivative was initially chosen because it is readily soluble in water.

To investigate the possibilities of a dynamically generated cyclodextrin phase, a test series of 22 products was examined on three different types of reversed-phase packing material. RP Select B (Merck), Hypersil BDS (Shandon) and Aluspher RP Select B (Merck) were selected as stationary phases. An aqueous solution containing 50 mM triethylamine and 50 mM of hydroxypropyl-β-cyclodextrin adjusted to pH 3 with sulfuric acid combined with methanol in an 80–20 volume ratio was used as the mobile phase.

Compared with the data obtained on a chemically bonded hydroxypropyl-β-cyclodextrin column using the same eluent, the chemically bonded column gives, in general, better results than the dynamically coated reversed-phase materials. Furthermore, alumina as stationary phase matrix seems to be less effective. However, in a few exceptional cases the Aluspher Select B column gives as good or even better results than the silica-based materials.

Cyclodextrin Phases in Preparative Chromatographic Applications

The importance of preparative chromatographic enantiomer separations in industry is continuously growing. Therefore it was very interesting to investigate the usefulness of cyclodextrin phases in preparative chromatographic applications.

On an experimental hydroxypropyl-β-cyclodextrin phase (Merck Darmstadt), several products which showed a good resolution on the corresponding analytical material were investigated on a preparative scale. An example of a preparative chromatographic separation is illustrated in **Figure 11**.

On the hydroxypropyl-β-cyclodextrin phase, in general a loading capacity of 2 mg g^{-1} of packing material was used. However, in some other cases we were able to load up to 4 mg of product per gram of stationary phase, which from a preparative chromatographic point of view certainly can be considered as a reasonable value for this type of application.

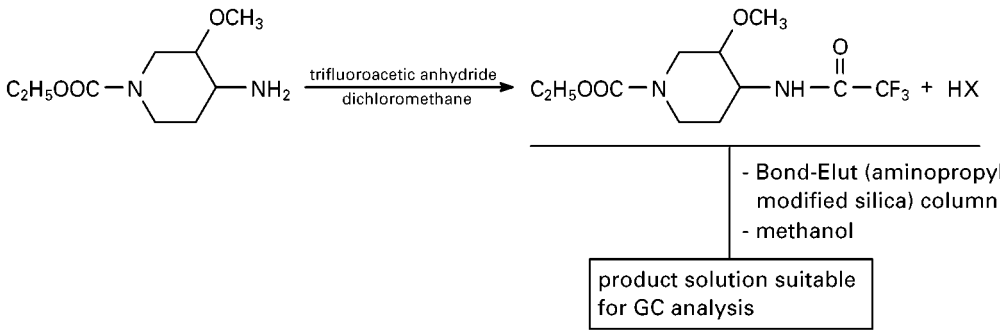
Cyclodextrins as Stationary Phases in Gas Chromatography

For the preparation of cyclodextrin-based capillary columns for gas chromatographic applications, two complementary methods have been developed and are commercially used. In the first method, alkylated cyclodextrins are diluted with polysiloxanes and immobilized on the inside wall of a fused silica capillary. In the other method, pentyl and hydroxyalkyldimethylcyclodextrins are coated on the inside wall of a fused silica capillary. Different well-known chromatography companies offer cyclodextrin-based capillary columns:

- Chrompack: diluted cyclodextrins;
- Macherey-Nagel: undiluted n-pentylated or acylated cyclodextrins;
- Advanced Separation Technologies: a broad variety of undiluted cyclodextrin derivatives (permethylated, hydroxypropyl, trifluoroacylated, butyrylated, dialkylated).

Different types of compounds (amines, epoxides, alkanes, alcohols, lactones, sugars, etc.) can be separated on cyclodextrin-based capillary columns.

The usefulness of these materials is illustrated by means of the separation of the four isomers of a piperidine derivative. Gas chromatography on cyclodextrin-based columns was tried after acetylation with trifluoracetic anhydride using the following procedure:



An advantage of this dervatization procedure was that all types of salts (used to investigate the possibilities of diastereomeric salt formation as a stereoselective synthesis method) could be acylated without prior liberation of the free base.

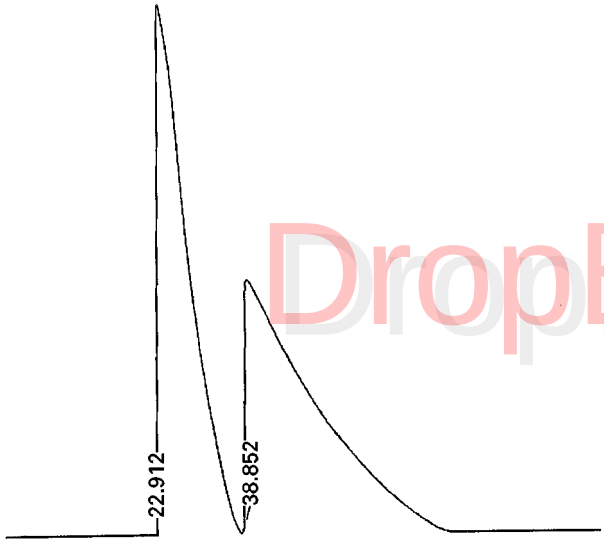
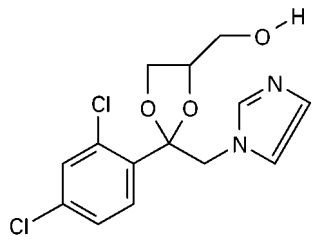


Figure 11 Preparative chromatographic separation on a β-cyclodextrin columns. Experimental condition: column: 80 mm ID dynamic axial compression column (Prochrom); stationary phase: 500 g 10 μm chemically bonded hydroxypropyl-β-cyclodextrin (experimental phase Merck Darmstadt; packing pressure 80 bar; mobile phase: 50 mM triethylamine adjusted with 50 mM sulfuric acid to pH 2.5–methanol (80–20, v/v); flow rate: 150 mL min^{-1}; detection: UV, wavelength 220 nm, range 2.56 AUFS; sample size: 1 g dissolved in 50 mL of concentrated sulfuric acid; solute:



(*trans*)

The first experiments were done on a 15 m × 0.32 mm ID Chiralsil-Dex® column (Chrompack). On this type of column, it was only possible to separate the *cis* and *trans* isomers.

Thereafter, chromatographic experiments were performed on four different cyclodextrin phases from Advanced Separation Technologies:

- Chiraldex® B-PH: (*S*)-2-hydroxypropyl permethylated β-cyclodextrin;
- Chiraldex® B-DA: (2,6-di-*O*-n-pentyl)-β-cyclodextrin;
- Chiraldex® B-TA: (2,6-di-*O*-n-pentyl-3-*O*-trifluoroacetyl)-β-cyclodextrin;
- Chiraldex® G-TA: (2,6-di-*O*-n-pentyl-3-*O*-trifluoroacetyl)-γ-cyclodextrin.

Chromatograms of the different experiments are illustrated in **Figure 12** and **Figure 13**. The largest differentiation between *cis* and *trans* isomers was observed on the permethylated hydroxypropyl-β-cyclodextrin column (Chiraldex® B-PH). However, the best separation of all isomers individually was observed on the trifluoroacylated β-cyclodextrin column (Chiraldex® B-TA).

Noticeable is the time required to perform an analysis. Compared with the analysis method on the crown ether column, a GC analysis is more than six times faster, although one has to take into account that on the crown ether column the diastereomeric salt could be analysed as such, while for the gas chromatographic method the product has to be derivatized prior to chromatography.

Cyclodextrins in Capillary Electrophoresis

In the pharmaceutical industry the importance of capillary electrophoresis is continuously growing. It is a technique which is increasingly used for determination of the optical purity of intermediates and final products. Many different optically pure compounds

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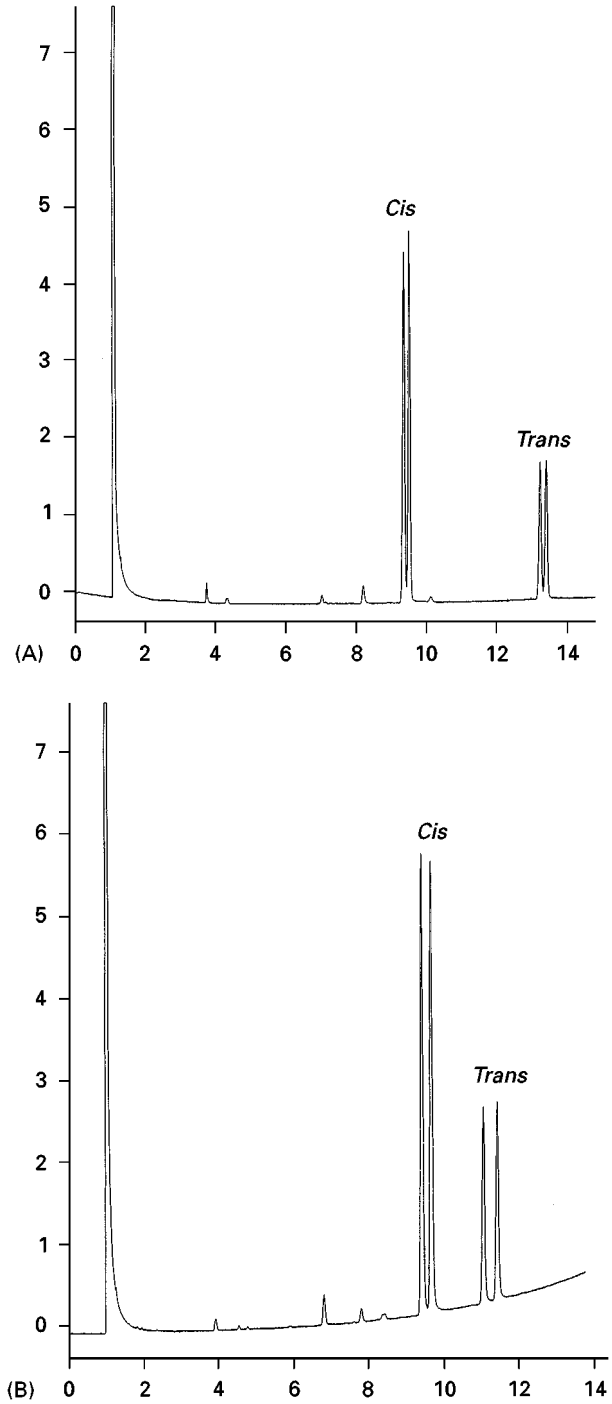

Figure 12 Capillary GC analysis on cyclodextrin stationary phases. Experimental conditions: column: (A) 15 m × 0.32 mm ID Chiraldex® B-PH ((*S*)-hydroxypropyl-β-cyclodextrin (Astec)); (B) 15 mm × 0.32 mm ID Chiraldex® B-DA (dipentylated β-cyclodextrin (Astec)); carrier gas: helium (linear velocity 25 cm s^{-1}); temperatures: column 150–200°C (3°C min^{-1}), injector 210°C, detector 210°C; detection: FID; injection: 1 μL split (split ratio 1/100).

can be used to generate the required chiral environment. Cyclodextrins of course are also very suitable as an electrolyte additive.

The different types of cyclodextrins, which are most frequently used are:

- α-cyclodextrin;
- β-cyclodextrin;
- γ-cyclodextrin;
- (2-hydroxy)propylated β-cyclodextrin;
- (2-hydroxy)propylated γ-cyclodextrin;
- Heptakis (2,6-di-*O*-methyl) β-cyclodextrin;
- Heptakis (2,3,6-tri-*O*-methyl) β-cyclodextrin;
- carboxymethylated β-cyclodextrin.

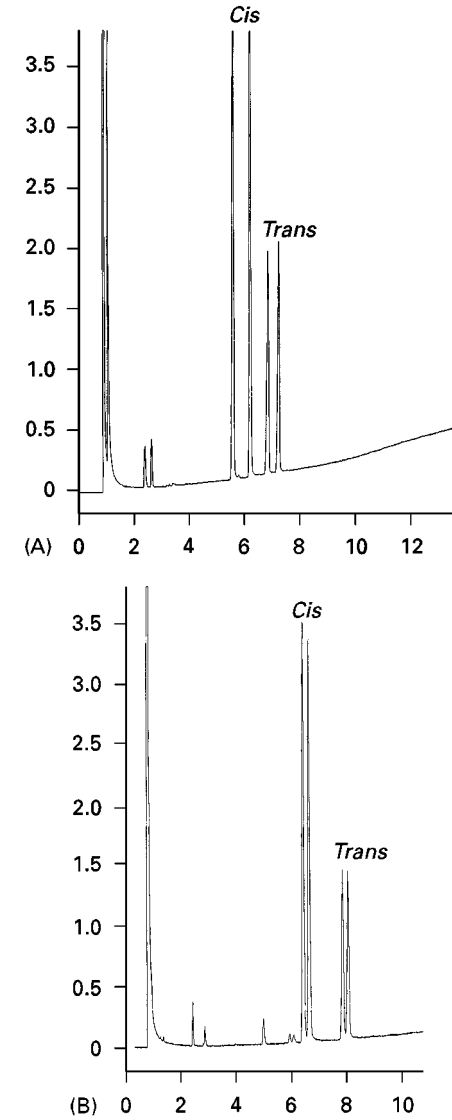

Figure 13 Capillary GC analysis on cyclodextrin stationary phases. Experimental conditions: column: (A) 15 m × 0.32 mm ID Chiraldex® B-TA (trifluoroacetyl-β-cyclodextrin (Astec)); (B) 15 m × 0.32 m ID Chiraldex® G-TA (trifluoroacetyl-γ-cyclodextrin (Astec)); carrier gas: helium (linear velocity 25 cm s^{-1}); temperatures: column 150–200°C (2°C min^{-1}), injector 210°C, detector 210°C; detection: FID; injection: 1 μL split (split ratio 1/100).

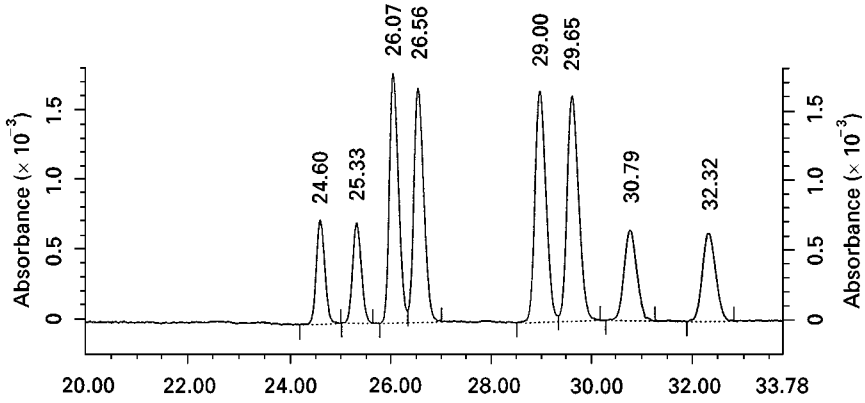

Figure 14 Capillary electrophoresis using a derivatized cyclodextrin as electrolyte additive. Experimental conditions: equipment: P/Ace system 5500 (Beckman); capillary: 50 μm ID uncoated fused silica; total length: 57 cm; length to detector: 50 cm; electrolyte: 20 mM Heptakis (2,3,6-tri-*O*-methyl)*β*-cyclodextrin 10 mM disodium hydrogen phosphate solution adjusted to pH 2.2 with phosphoric acid; analysis: temperature 25°C, voltage + 20 kV, inject sample 2 s, detection UV (220 nm).

The usefulness of cyclodextrins as an electrolyte additive is illustrated in the following example. A substance containing three optical centres, which means eight possible isomers, had to be separated. HPLC experiments on different types of chiral stationary phases did not succeed in a complete resolution of the mixture. The result of a capillary electrophoresis experiment using Heptakis (2,3,6-tri-*O*-methyl) *β*-cyclodextrin as electrolyte additive is illustrated in **Figure 14**.

Compared with HPLC, in capillary electrophoresis many more parameters can be varied to improve separation. Therefore, most of the methods developed on one of the commonly used chiral stationary phases can be replaced by a capillary electrophoresis methods, using cyclodextrins or another chiral auxiliary as electrolyte additive.

See also: **II/Chromatography: Liquid:** Ion Pair Liquid Chromatography; Mechanisms: Chiral; **III/Chiral Separations:** Amino Acids and Derivatives; Capillary Electrophoresis; Cellulose and Cellulose Derived Phases; Chiral Derivatization; Gas Chromatogrpahy; Ion-Pair Chromatography; Ligand Exchange Chromatography; Liquid Chromatography; Molecular Imprints as Stationary Phases; Protein Stationary Phases; Synthetic Multiple Interaction ('Pirkle') Stationary Phases; Thin-Layer (Planar) Chromatography.

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DropBooks

Gas Chromatography

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Introduction

The separation of enantiomers (optical isomers) by capillary gas chromatography on a chiral stationary phase (CSP) was discovered by Gil-Av and coworkers at the Weizmann Institute of Science, Israel, in 1966. At the outset of this work, according to Gil-Av,

> this topic was in a 'state of frustration'. Nobody believed it could be done. In fact, people were